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# Structural characterization of glucosylated GOS derivatives synthesized by the *Lactobacillus reuteri* GtfA and Gtf180 glucansucrase enzymes



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## ABSTRACT

β-Galacto-oligosaccharides (GOS) are used commercially in infant nutrition, aiming to functionally replace human milk oligosaccharides (hMOS). Glucansucrases Gtf180-ΔN and GtfA-ΔN of *Lactobacillus reuteri* strains convert sucrose into α-glucans with (α1→6)/(α1→3) and (α1→4)/(α1→6) glucosidic linkages, respectively. Previously we reported that both glucansucrases glucosylate lactose, producing a minimum of 5 compounds (degree of polymerization 3–4) (GL34 mixture) with (α1→2/3/4) linkages. This GL34 mixture exhibited growth stimulatory effects on various probiotic bacteria. Aiming to obtain additional compounds mimicking hMOS in structure and function, we here studied glucosylation of 3 commercially available galactosyl-lactose GOS compounds. Both Gtf180-ΔN and GtfA-ΔN were unable to use 3'-galactosyl-lactose (β3'-GL), but used sucrose to add a single glucose moiety to 4'-galactosyl-lactose (β4'-GL) and 6'-galactosyl-lactose (β6'-GL). β6'-GL was elongated at its reducing glucosyl unit with an (α1→2)-linked moiety and at its non-reducing end with an (α1→4) linked moiety; β4'-GL was only elongated at its reducing end with an (α1→2) linked moiety. Glucansucrases Gtf180-ΔN and GtfA-ΔN thus can be used to produce galactosyl-lactose-derived oligosaccharides containing (α1→2) and (α→4) glucosidic linkages, potentially with valuable bioactive (prebiotic) properties.

## 1. Introduction

The beneficial effects on human and animal health of so called prebiotics, a class of bioactive oligosaccharides, are widely studied [1–4]. A prebiotic is defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [5]. The most well-known prebiotics to date are human milk oligosaccharides (hMOS), β-galactooligosaccharides (GOS), β-fructooligosaccharides (FOS) and inulin, and lactulose [6–8]. Novel non-digestible carbohydrates and prebiotic compounds are still high in demand for food and feed product applications [9]. Recently, lactose-derived oligosaccharides have been at the focus of attention. The dominant members are GOS, with (β1→2), (β1→3), (β1→4), or (β1→6) linked galactosyl moieties, of various sizes (mostly DP2–DP5). These prebiotic compounds stimulate growth of probiotic bacteria to various extents [7,10,11]. Lactosucrose and lactulose are also well studied for their selective stimulatory effects on human gut bacteria [12,13].

Glucansucrases belong to glycoside hydrolase family 70 (GH70) (<http://www.CAZy.org>) and are extracellular trans-glycosidases found in lactic acid bacteria [14,15]. Glucansucrase catalyzed reactions with

sucrose follow an α-retaining double-displacement mechanism. Depending on the nature of the acceptor substrate, glucansucrase enzymes catalyze three types of reactions: hydrolysis of sucrose with water as acceptor, polymerization with growing α-glucan chains as acceptor, or trans-glycosylation with sucrose as donor substrate and other compounds as acceptor substrates (including oligosaccharides) [16]. Depending on their specificity, glucansucrases are capable of producing α-glucans with various linkage types, namely (α1→3), (α1→4) and (α1→6). Only the branching glucansucrase Dsr-E from *Leuconostoc mesenteroides* NRRLB-1299 can introduce single (α1→2) glucosyl branches in a dextran backbone [17,18]. The *Lactobacillus reuteri* Gtf180-ΔN and GtfA-ΔN enzymes produce α-glucans with 69% (α1→6) and 31% (α1→3) glycosidic linkages and 58% (α1→4) and 42% (α1→6) glycosidic linkages, respectively [19,20]. Glucansucrases are known for their ability to use a wide variety of acceptor substrates including oligosaccharides and non-glycan compounds [21–23]. One example is use of maltose as acceptor substrate resulting in synthesis of various longer oligosaccharides [15,24,25]. Recently, lactose has attracted interest as acceptor substrate for glucansucrase enzymes. Dextransucrases from *Leuconostoc*

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*mesenteroides* and *Weissella confusa* added a single glucose moiety to lactose, involving an ( $\alpha 1 \rightarrow 2$ ) linkage, to synthesize 2- $\alpha$ -D-glucopyranosyl-lactose [26,27]. Lately, we reported that Gtf180- $\Delta$ N and GtfA- $\Delta$ N synthesize a mixture of five new glucosylated lactose derivatives (GL34), using sucrose as donor substrate and lactose as acceptor substrate [28]. A study of the potential prebiotic properties of GL34 revealed selective stimulatory effects on growth of various bifidobacteria. Structural analysis showed that these compounds are lactose elongated with one or two glucose moieties (DP3 - DP4) involving ( $\alpha 1 \rightarrow 2$ ), ( $\alpha 1 \rightarrow 3$ ), ( $\alpha 1 \rightarrow 4$ ) glycosidic linkages [29]. With lactose as acceptor substrate, Gtf180- $\Delta$ N and GtfA- $\Delta$ N thus produced oligosaccharide compounds with new linkage types, ( $\alpha 1 \rightarrow 2$ ) and ( $\alpha 1 \rightarrow 4$ ) for Gtf180- $\Delta$ N and ( $\alpha 1 \rightarrow 2$ ) and ( $\alpha 1 \rightarrow 3$ ) for GtfA- $\Delta$ N, not observed for these glucansucrases before. The high resistance of this ( $\alpha 1 \rightarrow 2$ )-linkage type to human digestive enzymes and their selective stimulatory effects on probiotic bacteria make ( $\alpha 1 \rightarrow 2$ ) linkage-containing oligosaccharides strong candidates for new non-digestible carbohydrates and prebiotic ingredients [30].

In view of these interesting findings, we aimed to further exploit the ability of glucansucrases Gtf180- $\Delta$ N and GtfA- $\Delta$ N using the commercially available DP3 GOS structures 3'-galactosyl-lactose ( $\beta 3'$ -GL), 4'-galactosyl-lactose ( $\beta 4'$ -GL) and 6'-galactosyl-lactose ( $\beta 6'$ -GL), as acceptor substrates to synthesize new (bioactive) oligosaccharides. The transfer products were structurally analyzed by highperformance anion-exchange chromatography (HPAEC), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and 1D/2D  $^1\text{H}/^{13}\text{C}$  nuclear magnetic resonance (NMR) spectroscopy (TOCSY, ROESY). Three new DP4 structures were identified with ( $\alpha 1 \rightarrow 2$ ) and ( $\alpha 1 \rightarrow 4$ ) glycosidic linkages.

## 2. Results

### 2.1. Trans-glucosylation of GOS

$\beta 3'$ -GL,  $\beta 4'$ -GL and  $\beta 6'$ -GL (0.02 M) were incubated with sucrose

(0.05 M) plus the Gtf180- $\Delta$ N or GtfA- $\Delta$ N enzymes, at 37 °C and pH 4.7. Blank reactions minus  $\beta$ -GL compounds used sucrose as both acceptor and donor substrate, resulting in  $\alpha$ -glucan synthesis. Incubation mixtures were sampled after 0 h, 5 h and 24 h, and subjected to HPAEC-PAD analysis (Fig. 1). No trans-glucosylation products were observed with  $\beta 3'$ -GL (data not shown). Gtf180- $\Delta$ N and GtfA- $\Delta$ N incubated with sucrose plus  $\beta 6'$ -GL yielded similar HPAEC-PAD profiles of oligosaccharides synthesized (Fig. 1a and c), with three major peaks at retention times between 22 and 29 min. In case of Gtf180- $\Delta$ N, there are several minor peaks eluting later in time, which most likely are higher DP oligosaccharides (DP5 - DP9). The areas of the Gtf180- $\Delta$ N peaks at 23.0 min and 25.5 min, called GL1 and GL2 respectively, are much more significant than those from GtfA- $\Delta$ N. Regarding the glucosylation of  $\beta 4'$ -GL, one significant peak at retention time of 31.9 min, called GL3, was observed in HPAEC-PAD profiles of the reaction mixtures of both Gtf180- $\Delta$ N and GtfA- $\Delta$ N (Fig. 1b and d). Especially with Gtf180- $\Delta$ N, there are several minor peaks that elute later than GL3. Similarly, the intensity of peak GL3 in the profile of Gtf180- $\Delta$ N is much more significant than that of GtfA- $\Delta$ N, especially after 5 h of incubation. The areas of the peaks GL1, GL2 and GL3, decreased upon prolonged incubation. This may be due to further glucansucrase catalyzed elongation reactions using these trans-glucosylation products as intermediate acceptor substrates. Further studies involving structural elucidation of the higher DP trans-glucosylation products are required to fully understand this. In case of Gtf180- $\Delta$ N,  $\beta 6'$ -GL and  $\beta 4'$ -GL were converted for 26 and 32% (Fig. 1a and b; 24 h incubation time), estimated from their peak areas. Only limited amounts of  $\beta 6'$ -GL and  $\beta 4'$ -GL were available, and further optimizations of reaction conditions and product yields remain to be done.

### 2.2. Structural analysis of trans-glucosylation products

The three major glucosylation products corresponding to the peaks GL1, GL2 and GL3 of Gtf180- $\Delta$ N decorating  $\beta 6'$ -GL and  $\beta 4'$ -GL (Fig. 1) were isolated from the incubation mixture for structural analysis by

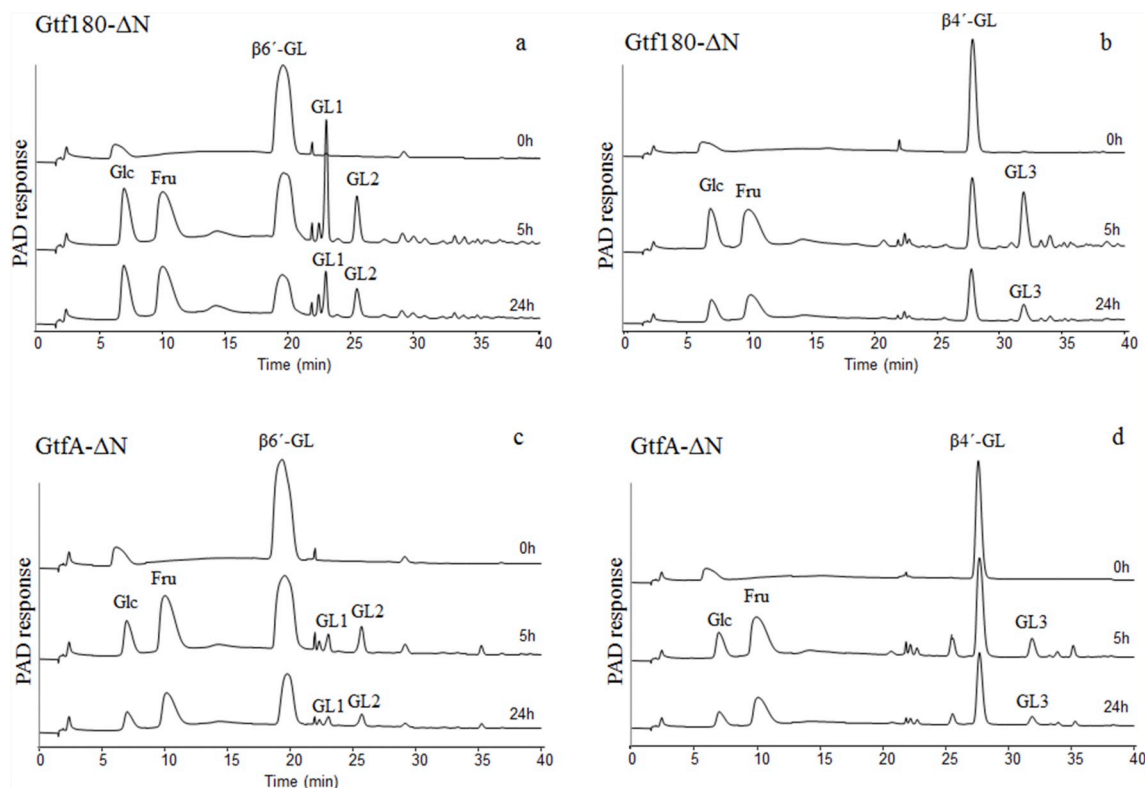
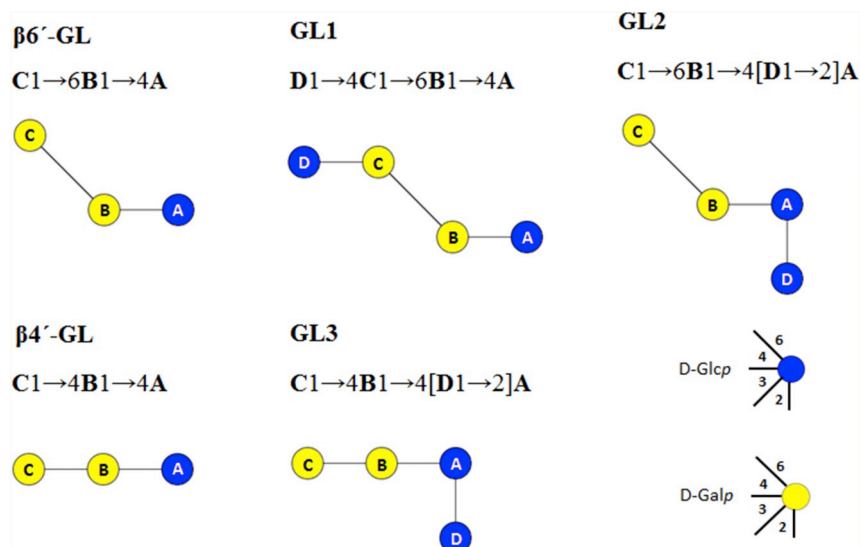


Fig. 1. HPAEC-PAD chromatograms of the reaction product mixtures obtained with 3 U mL<sup>-1</sup> glucansucrases (Gtf180- $\Delta$ N and GtfA- $\Delta$ N) using a)  $\beta 6'$ -GL and b)  $\beta 4'$ -GL as acceptor substrate and sucrose as donor substrate, after various times of incubation at 37 °C and pH 4.7.



**Scheme 1.** Structures of the acceptor substrates  $\beta 6'$ -GL and  $\beta 4'$ -GL and the GL1-GL3 transfer products synthesized by Gtf180- $\Delta$ N and GtfA- $\Delta$ N using sucrose as donor substrate.

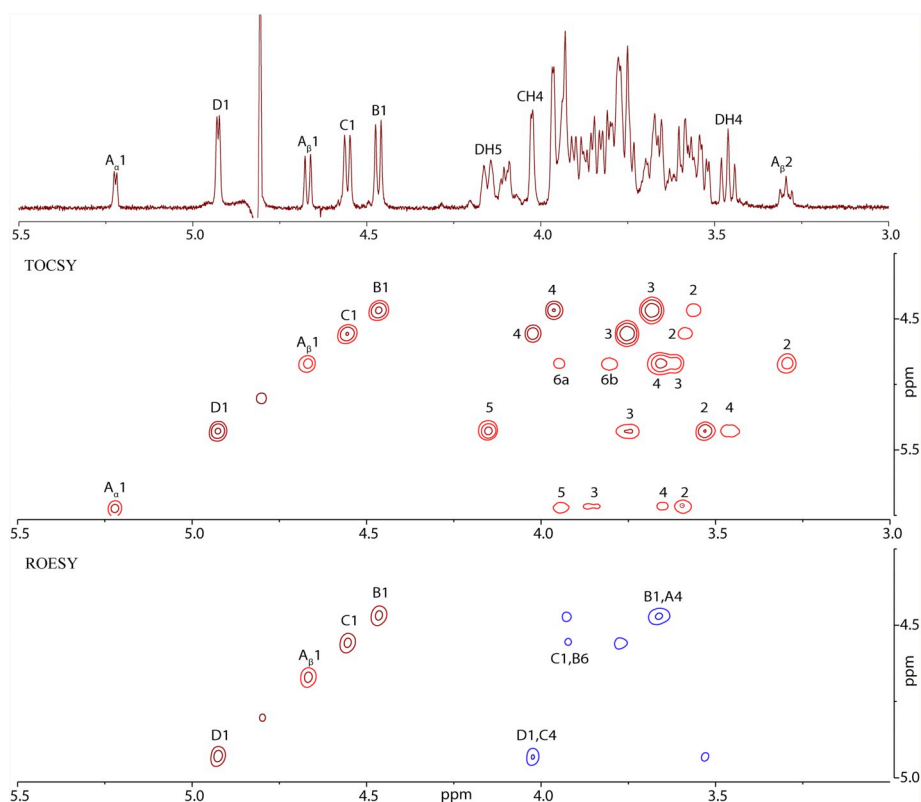
MALDI-TOF-MS and 1D/2D  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy. The purity and retention time of each fraction was confirmed by reinjection on an analytical CarboPac PA-1 ( $4 \times 250$  mm) column. The fragment size distribution of each fraction was determined by MALDI-TOF MS. The data showed that all three major products corresponded to tetrasaccharides, as evidenced by a pseudo-molecular sodium adduct ion at 689 m/z.

### 2.3. Glucosylated transfer products of $\beta 6'$ -GL

#### 2.3.1. Fraction GL1

Tetrasaccharide GL1 includes 4 hexose residues, namely **A**, **B** and **C**

(one glucosyl and two galactosyl residues from the  $\beta 6'$ -GL substrate), and **D** (transferred glucosyl residue from sucrose) (Scheme 1). The 1D  $^1\text{H}$  NMR spectrum of this fraction exhibited five anomeric signals at  $\delta$  5.222 (**A**<sub>a</sub> H-1),  $\delta$  4.670 (**A**<sub>B</sub> H-1),  $\delta$  4.468 (**B** H-1),  $\delta$  4.55 (**C** H-1) and  $\delta$  4.927 (**D** H-1) (Fig. 2). All non-anomeric proton resonances were assigned by using 2D  $^1\text{H}$ - $^1\text{H}$  TOCSY and 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC (Table 1 and Fig. 2). Residue **A** showed the  $^1\text{H}$  and  $^{13}\text{C}$  pattern fitting with a reducing residue. Moreover, the set of chemical shifts of this residue matches very well with the corresponding residue from the structure  $\beta 6'$ -GL [31]. These data show that there is no other substitution at residue **A** except for the 4-substitution with the galactosyl residue **B**. There are



**Fig. 2.** 500-MHz 1D  $^1\text{H}$  NMR spectra of GL1 synthesized by Gtf180- $\Delta$ N from  $\beta 6'$ -GL, recorded at 25 °C in  $\text{D}_2\text{O}$ . Anomeric signals of each fraction were labeled according to the legends of the corresponding structures indicated in Scheme 1. The  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of GL1 are listed in Table 1.

**Table 1**

<sup>1</sup>H and <sup>13</sup>C chemical shifts of the glucosylated-β6'-GL derivatives GL1 and GL2, measured at 25 °C in D<sub>2</sub>O. Chemical shifts that are key in the structural determination are underlined.

	β6'-GL		GL1		GL2	
	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C
A <sub>α</sub> 1	5.223	93.1	5.222	92.5	5.447	89.6
A <sub>α</sub> 2	3.57	72.1	3.60	71.7	<u>3.72</u>	<u>80.0</u>
A <sub>α</sub> 3	3.83	72.8	3.85	72.8	3.99	71.0
A <sub>α</sub> 4	<u>3.63</u>	<u>80.2</u>	<u>3.66</u>	<u>80.2</u>	<u>3.68</u>	<u>80.8</u>
A <sub>α</sub> 5	3.95	71.1	3.96	69.5	3.98	69.2
A <sub>α</sub> 6a	3.88	61.1	3.88	60.8	3.88	61.0
A <sub>α</sub> 6b	3.84		3.84	61.0	3.84	61.2
A <sub>β</sub> 1	4.667	96.9	4.670	96.4	4.527	104.3
A <sub>β</sub> 2	3.294	74.9	3.296	74.5	<u>3.45</u>	<u>78.8</u>
A <sub>β</sub> 3	3.63	75.8	3.63	75.5	3.75	74.0
A <sub>β</sub> 4	<u>3.65</u>	<u>80.2</u>	<u>3.65</u>	<u>79.8</u>	<u>3.69</u>	<u>80.8</u>
A <sub>β</sub> 5	3.60	75.9	3.62	75.5	3.61	75.4
A <sub>β</sub> 6a	3.94	61.4	3.94	60.9	3.97	61.2
A <sub>β</sub> 6b	3.80		3.80	60.9	3.81	
B1	4.483	104.4	4.468	103.8	4.468/4.483	104.3
B2	3.53	72.0	3.56	72.2	3.57	71.8
B3	3.66	73.8	3.68	73.2	3.68	73.5
B4	3.94	69.7	3.96	69.4	3.98	69.3
B5	3.95	75.0	3.93	75.0	3.92	74.6
B6a	<u>4.079</u>	<u>70.3</u>	<u>4.10</u>	<u>69.8</u>	<u>4.06</u>	<u>69.8</u>
B6b	<u>3.93</u>		<u>3.94</u>		<u>3.90</u>	<u>69.8</u>
C1	4.460	104.4	<u>4.55</u>	104.1	4.441/4.454	104.3
C2	3.54	72.0	3.60	71.7	3.54	72.4
C3	3.67	73.8	3.76	73.2	3.66	73.4
C4	3.974	69.7	<u>4.02</u>	<u>77.9</u>	3.92	69.6
C5	3.68	76.3	3.78	76.0	3.70	75.9
C6a	3.81		3.81	60.8	3.81	61.2
C6b	3.76		3.79	60.8	3.77	61.8
D <sup>α</sup> 1			4.927	100.8	5.127	96.5
D <sup>α</sup> 2			3.54	72.8	3.55	71.6
D <sup>α</sup> 3			3.75	72.0	3.86	<u>73.4</u>
D <sup>α</sup> 4			3.46	69.8	3.45	70.3
D <sup>α</sup> 5			4.152	72.7	3.96	72.3
D <sup>α</sup> 6a					3.91	61.0
D <sup>α</sup> 6b					3.80	61.0
D <sup>β</sup> 1					5.428	98.3
D <sup>β</sup> 2					3.54	72.4
D <sup>β</sup> 3					<u>3.78</u>	<u>73.6</u>
D <sup>β</sup> 4					3.46	70.3
D <sup>β</sup> 5					4.03	60.9
D <sup>β</sup> 6a					3.82	
D <sup>β</sup> 6b						

downfield shifts detected in residue C H-2 at δ 3.76 (Δδ + 0.09 ppm), H-3 at δ 4.02 (Δδ + 0.05 ppm), H-4 at 4.02 (Δδ + 0.05 ppm) and H-5 at δ 3.78 (Δδ + 0.10 ppm), which are indicative for substitution occurring at this residue. The residue C substitution was at C-4 as evidenced by the strong downfield shift to δ 77.9 (Δδ + 8.2 ppm). This 4-substitution was supported by the ROESY inter-residual correlations between D H-1 and C H-4 (Fig. 2). Other inter-residual interactions between B H-1 and A H-4 and between C H-1 and B H-6 were also detected in the ROESY spectrum. These data lead to identification of the GL1 structure as α-D-Glcp-(1 → 4)-D-Galp-(1 → 6)-β-D-Galp-(1 → 4)-D-Glcp; D1 → 4C1 → 6B1 → 4A (Scheme 1).

## 2.4. Fraction GL2

Tetrasaccharide GL2 includes 4 hexose residues, namely A, B and C (one glucosyl and two galactosyl residues from the β6'-GL substrate), and D (transferred glucosyl residue from sucrose) (Scheme 1). The 1D <sup>1</sup>H NMR of fraction GL2 showed anomeric peaks at δ 5.447 (A<sub>α</sub> H-1), δ 4.572 (A<sub>β</sub> H-1), δ 4.48 (B H-1), δ 4.448 (C H-1), δ 5.172 (D<sup>α</sup> H-1) and

5.428 (D<sup>β</sup> H-1) (Table 1). The anomeric signals B H-1 and C H-1 are split under influence of the reducing residue A anomeric configuration. This splitting is typically observed as a result of a 2-substitution of residue A [13]. All other <sup>1</sup>H and <sup>13</sup>C chemical shifts of this structure were assigned from 2D NMR spectra (COSY, TOCSY, ROESY and HSQC) (Fig. 3). Residue A<sub>α</sub> and A<sub>β</sub> H-1 at δ 5.447 and δ 4.527, respectively, are shifted very strongly compared with β6'-GL, reflecting a substituted reducing α-D-Glcp residue at C-2 [32]. Furthermore, the A<sub>α</sub> H-2/C-2 at δ 3.70/80.0 ppm and A<sub>β</sub> H-2/C-2 at δ 3.45/78.8 ppm indicates a 2-substituted residue. The 2-substitution of residue A is further confirmed by inter-residual ROESY correlations between D<sup>α</sup> H-1/A<sub>α</sub> H-2 and D<sup>β</sup> H-1/A<sub>β</sub> H-2 (Fig. 3). Residue D showed the chemical shift pattern of a terminal α-D-Glcp residue with D<sup>α</sup>/D<sup>β</sup> H-4 at δ 3.45/3.46 and H-1 at δ 5.127/5.428 [32]. Residue C showed H-2, H-3 and H-4 at δ 3.54, δ 3.66 and δ 3.92 matching with the terminal (1 → 6)-linked galactosyl residue [32]. Moreover, ROESY inter-residual correlations were observed between C H-1 and B H-6 and between B H-1 and A H-4, confirming the β-D-Galp-(1 → 6)-β-D-Galp-(1 → 4)-D-Glcp-. These data resulted in identification of the GL2 structure as β-D-Galp-(1 → 6)-β-D-Galp-(1 → 4)-[α-D-Glcp-(1 → 2)]-D-Glcp; C1 → 6B1 → 4[D1 → 2]A (Scheme 1).

## 2.5. Glucosylated transfer product of β4'-GL

### 2.5.1. Fraction GL3

Tetrasaccharide GL3 comprises of 4 hexose residues, namely A, B and C (one glucosyl and two galactosyl residues from β4'-GL), and D (transferred glucosyl residue from sucrose) (Scheme 1). The 1D <sup>1</sup>H NMR spectrum of GL3 revealed six anomeric <sup>1</sup>H signals at δ 5.435 (A<sub>α</sub> H-1), δ 4.817 (A<sub>β</sub> H-1), δ 4.489 (B H-1), δ 4.594 (C H-1), δ 5.093 (D<sup>α</sup> H-1) and δ 5.355 (D<sup>β</sup> H-1) (Table 2). The splitting of the anomeric signals B H-1 and C H-1 is influenced by the α/β configuration of the reducing residue A. From 2D NMR spectra <sup>1</sup>H and <sup>13</sup>C chemical shifts were determined for all residues (Fig. 4). The anomeric signals of residue C at δ 4.601/4.594 are indicative of a terminal β-D-Galp-(1 → 4)- residue [32], indicating that this residue had not become elongated. Whereas, residue A<sub>α</sub> and A<sub>β</sub> H-1 at δ 5.435 and δ 4.817, respectively, reflects a 2-substitution [32]. Also the A<sub>α</sub> H-2/C-2 at δ 3.69/79.3 ppm and A<sub>β</sub> H-2/C-2 at δ 3.41/79.0 ppm supports the occurrence of a 2-substitution. This (1 → 2)-linked residue is further confirmed by inter-residual ROESY correlations between D<sup>α</sup> H-1 and A<sub>α</sub> H-2 and between D<sup>β</sup> H-1 and A<sub>β</sub> H-2 (Fig. 4). The chemical shift pattern of residue D reflects a terminal α-D-Glcp [32]. The signal at δ 4.497/4.483 of residue B fits with a (→ 4)-β-D-Galp-(1 → 4)-D-Glcp residue. The ROESY spectrum also showed inter-residual correlations between C H-1 and B H-4 and between B H-1 and A H-4. Combining all data, the structure of tetrasaccharide compound GL3 is determined as β-D-Galp-(1 → 4)-β-D-Galp-(1 → 4)-[α-D-Glcp-(1 → 2)]-D-Glcp; C1 → 4B1 → 4[D1 → 2]A (Scheme 1).

## 3. Discussion and conclusions

Glucansucrases GtfA-ΔN and Gtf180-ΔN use sucrose to synthesize α-glucan polymers with (α1 → 4)/(α1 → 6) and (α1 → 6)/(α1 → 3) linkages, respectively [19,20]. Their linkage specificity is maintained in the acceptor reaction with maltose and other carbohydrates, or non-glycans such as catechol [21,22,33,34]. When acting on lactose as acceptor substrate, these two enzymes produced the same trans-glycosylation product mixture (GL34), with compounds F1 - F5 but introduced different linkage types, (α1 → 2)/(α1 → 4) for Gtf180-ΔN and (α1 → 2)/(α1 → 3) for GtfA-ΔN [28]. Also in the present study, when using galactosyl-lactose GOS molecules as acceptor substrate, these two enzymes synthesized similar products. Both GtfA-ΔN and Gtf180-ΔN produced α-D-Glcp-(1 → 4)-D-Galp-(1 → 6)-β-D-Galp-(1 → 4)-D-Glcp and β-D-Galp-(1 → 6)-β-D-Galp-(1 → 4)-[α-D-Glcp-(1 → 2)]-D-Glcp (GL1 and GL2) when acting on β6'-GL, and produced β-D-Galp-(1 → 4)-β-D-Galp-(1 → 4)-[α-D-Glcp-(1 → 2)]-D-Glcp (GL3) when acting on β4'-



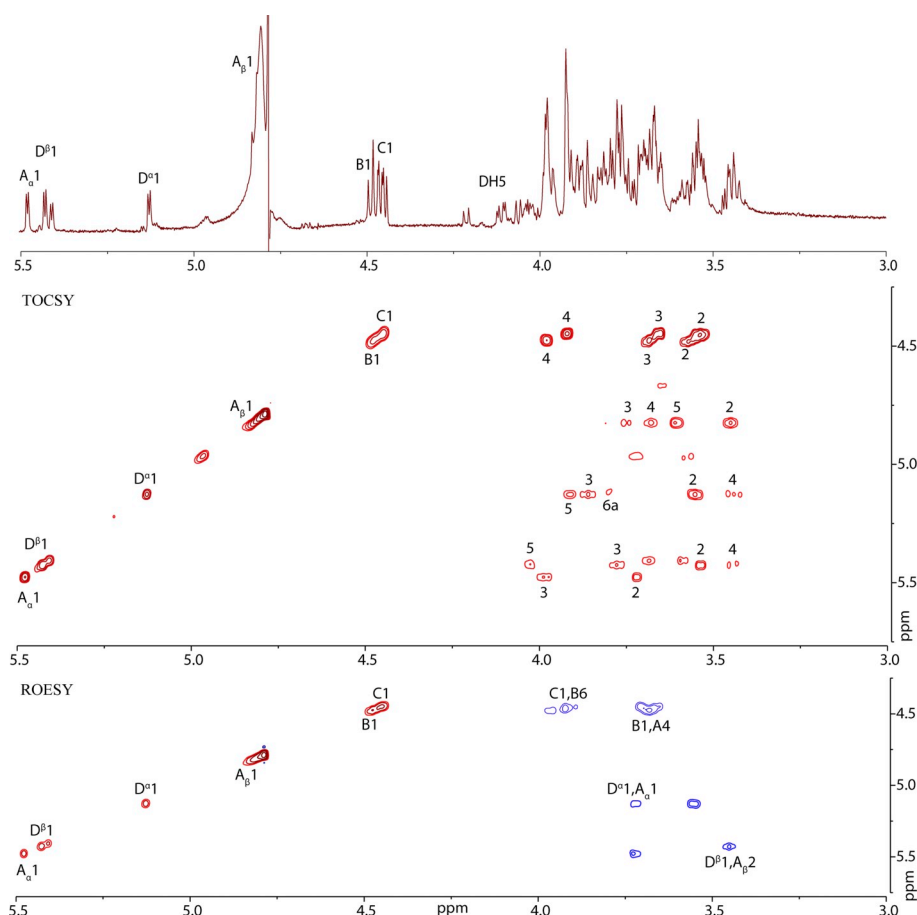


Fig. 3. 500-MHz 1D  $^1\text{H}$  NMR spectra of GL2 synthesized by Gtf180- $\Delta\text{N}$  from  $\beta 6'$ -GL, recorded at 25  $^\circ\text{C}$  in  $\text{D}_2\text{O}$ . Anomeric signals of each fraction were labeled according to the legends of the corresponding structures indicated in Scheme 1. The  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of GL2 are listed in Table 1.

GL (Scheme 1). Gtf180- $\Delta\text{N}$  produced significantly more of these products than GtfA- $\Delta\text{N}$  (Fig. 1). With  $\beta 6'$ -GL as acceptor substrate both enzymes introduced an  $(\alpha 1 \rightarrow 4)$  linkage at the non-reducing end in the GL1 product, but this is not the case for  $\beta 4'$ -GL. Both glucansucrases added a Glc-(1  $\rightarrow$  4) residue on  $\beta$ -Gal-(1  $\rightarrow$  4)-Glc of lactose and  $\beta$ -Gal-(1  $\rightarrow$  6)-Gal- of  $\beta 6'$ -GL but not on  $\beta$ -Gal-(1  $\rightarrow$  4)- $\beta$ -Gal of  $\beta 4'$ -GL. Different from lactose as acceptor substrate [28], none of the Gal-(1  $\rightarrow$  x)-Gal epitopes of  $\beta 6'$ -GL and  $\beta 4'$ -GL can be elongated with a Glc-(1  $\rightarrow$  3) residue. However, similar to the reaction with lactose [28], with  $\beta 6'$ -GL and  $\beta 4'$ -GL as acceptor substrates, both enzymes introduced an  $(\alpha 1 \rightarrow 2)$  linked glucose moiety at the reducing end in the GL2 and GL3 products, respectively (Scheme 1). Galactose-containing acceptor substrates thus appear to enforce changes in the glucoside linkage specificity of these two glucansucrases: Gtf180- $\Delta\text{N}$  and GtfA- $\Delta\text{N}$  favor the synthesis of  $(\alpha 1 \rightarrow 2)$  linkage containing oligosaccharides when acting on galactose-containing acceptor compounds. Both Gtf180- $\Delta\text{N}$  and GtfA- $\Delta\text{N}$  were unable to use  $\beta 3'$ -GL as acceptor substrate. The presence of a  $(\beta 1 \rightarrow 3)$  linkage in this acceptor compound probably makes it inaccessible to the acceptor binding site of the studied glucansucrases. Docking experiments with the  $\beta 4'$ -GL and  $\beta 6'$ -GL substrates and a glucosyl-enzyme intermediate constructed using the crystal structure of *L. reuteri* 180 Gtf180- $\Delta\text{N}$  (PDB: 3KLK; Vujičić-Žagar et al., 2010) resulted in different poses and were inconclusive. Further insights in the linkage specificity determinants of Gtf180- $\Delta\text{N}$  acting on these GOS-DP3 acceptor substrates may be provided by a crystal structure of Gtf180- $\Delta\text{N}$  in complex with lactose, but attempts to obtain such a complex have not been successful so far.

The acceptor substrates  $\beta 6'$ -GL and  $\beta 4'$ -GL used in this study are present in the well-known commercial prebiotic mixture Vivinal GOS

[32,35]. These two compounds previously showed selective stimulatory effects on growth of various beneficial bacteria including *Bifidobacterium breve*, *Bifidobacterium longum* subspecies *infantis*, *Bifidobacterium adolescentis*, *Bifidobacterium longum* subspecies *longum*, *Bifidobacterium lactis* and *Lactobacillus acidophilus* [36] and [M. Boger. et al. in preparation]. However, the linear structure  $\beta 4'$ -GL also was readily digested by commensal bacteria like *Bacteroides thetaiotaomicron* and other bacteria encoding endo- $\beta$ -galactanase GH53 enzymes [37]. Elongation of  $\beta 4'$ -GL with an  $(\alpha 1 \rightarrow 2)$  linked glucose moiety may improve its resistance towards consumption by commensal bacteria, and may promote its non-digestible and prebiotic properties. The  $\beta 6'$ -GL compound was not digested by commensal bacteria and predicted to stimulate growth of beneficial gut bacteria in a similar manner as hMOS [37]. Glucosylation of  $\beta 6'$ -GL may even further enhance its selectivity and thus provides another hMOS mimicking compound. The potential stimulatory effects of these new GL1-GL3 DP4 compounds on growth of probiotic bacteria, and other functional properties, remain to be studied. Optimization of the reaction conditions, to enhance galactosyl-lactose conversion and product yields, are required to obtain sufficient amounts of the GL1-GL3 compounds for such functional studies. Trans-glucosylation of galactosyl-lactose compounds with glucansucrase enzymes is likely to further expand the already well-known prebiotic GOS status.

## 4. Experimental section

### 4.1. Glucansucrase enzymes

*Escherichia coli* BL21 (DE3) (Invitrogen) carrying plasmid pET15b

**Table 2**

$^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of the glucosylated- $\beta$ 4'-GL derivative GL3, measured at 25 °C in  $\text{D}_2\text{O}$ . Chemical shifts that are key in the structural determination are underlined.

	$\beta$ 4'-GL		GL3	
	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$
A $_{\alpha}$ 1	5.225	92.9	5.435	90.1
A $_{\alpha}$ 2	3.58	<u>72.6</u>	<u>3.69</u>	<u>79.3</u>
A $_{\alpha}$ 3	3.83	72.6	3.94	70.7
A $_{\alpha}$ 4	3.65	79.5	<u>3.71</u>	<u>79.2</u>
A $_{\alpha}$ 5	3.95	71.1	3.95	70.7
A $_{\alpha}$ 6a	3.87	61.0		
A $_{\alpha}$ 6b	3.80			
A $_{\beta}$ 1	4.664	96.9	4.817	96.8
A $_{\beta}$ 2	3.281	74.9	<u>3.41</u>	<u>79.0</u>
A $_{\beta}$ 3	3.65	75.3	3.74	75.4
A $_{\beta}$ 4	3.65	79.5	<u>3.67</u>	<u>79.5</u>
A $_{\beta}$ 5	3.60	76.0	3.59	75.5
A $_{\beta}$ 6a	3.958	61.2	3.97	60.9
A $_{\beta}$ 6b	3.80		3.81	
B1	4.486	104.0	4.483/4.497	103.8
B2	3.63	72.4	3.64	72.2
B3	3.77	73.9	3.78	73.8
B4	4.193	78.3	<u>4.192</u>	<u>78.0</u>
B5	3.74	75.5	3.74	75.4
B6a	3.80	61.9	3.82	61.57
B6b	3.11			
C1	4.603	105.2	4.594/4.601	105.1
C2	3.57	72.4	3.59	75.6
C3	3.66	74.0	3.66	72.3
C4	3.904	69.8	3.905	69.4
C5	3.71	76.1	3.69	76.1
C6a	3.82	61.6	3.81	61.0
C6b	3.80			
D $^{\alpha}$ 1			5.093	97.4
D $^{\alpha}$ 2			3.55	72.3
D $^{\alpha}$ 3			3.80	73.7
D $^{\alpha}$ 4			3.45	70.1
D $^{\alpha}$ 5			3.98	72.5
D $^{\alpha}$ 6a			3.89	60.7
D $^{\alpha}$ 6b				
D $^{\beta}$ 1			5.355	98.7
D $^{\beta}$ 2			3.54	72.2
D $^{\beta}$ 3			3.75	73.7
D $^{\beta}$ 4			3.47	70.1
D $^{\beta}$ 5			4.077	72.3
D $^{\beta}$ 6a			3.91	60.9
D $^{\beta}$ 6b				

with the *gtf180* and *gtfA* genes from *Lactobacillus reuteri* strains 180 and 121 was used for expression of the N-terminally truncated glucansucrase enzymes (Gtf180- $\Delta$ N and GtfA- $\Delta$ N). The expression and purification of these glucansucrases have been described previously [22,38].

#### 4.2. Trans-glucosylation reactions

The total activity of purified Gtf180- $\Delta$ N or GtfA- $\Delta$ N was measured as initial rates with sucrose by methods described previously by Van Geel-Schutten et al. [39]. The products of the trans-glucosylation reaction were prepared by incubating a mixture of 0.05 M sucrose (donor substrate) and 0.02 M GOS (acceptor substrate) with 3 U mL $^{-1}$  glucansucrase at 37 °C in 50 mM sodium acetate buffer with 0.1 mM  $\text{CaCl}_2$  at pH 4.7. A volume of 10  $\mu\text{L}$  of the reaction mixtures was taken at 0 h, 5 h and 24 h and then mixed with 190  $\mu\text{L}$  DMSO. The diluted samples were analyzed by High-pH anion-exchange chromatography (HPAEC-PAD).

#### 4.3. Isolation and purification of oligosaccharide products

The reactions were carried out in a volume of 20 mL under the conditions described in the previous section of Trans-glucosylation reactions. Afterwards the reaction mixtures were diluted with two volumes of cold ethanol 20% and stored at 4 °C overnight to precipitate any polysaccharide material present. After centrifugation at 10,000 g for 10 min, the supernatants were applied to a rotatory vacuum evaporator to remove ethanol. The aqueous fractions were then absorbed onto a CarboGraph SPE column (GRACE, USA) using acetonitrile:water = 1:3 as eluent, followed by evaporation of acetonitrile under an  $\text{N}_2$  stream before being freeze-dried. This was followed by fractionation HPAEC on a Dionex ICS-5000 workstation (Dionex, Amsterdam, the Netherlands), equipped with a CarboPac PA-1 column (250  $\times$  9 mm; Dionex) and an ED40 pulsed amperometric detector (PAD). The collected fractions were neutralized by acetic acid 20% and then desalted using a CarboGraph SPE column as described earlier.

#### 4.4. HPAEC-PAD analysis

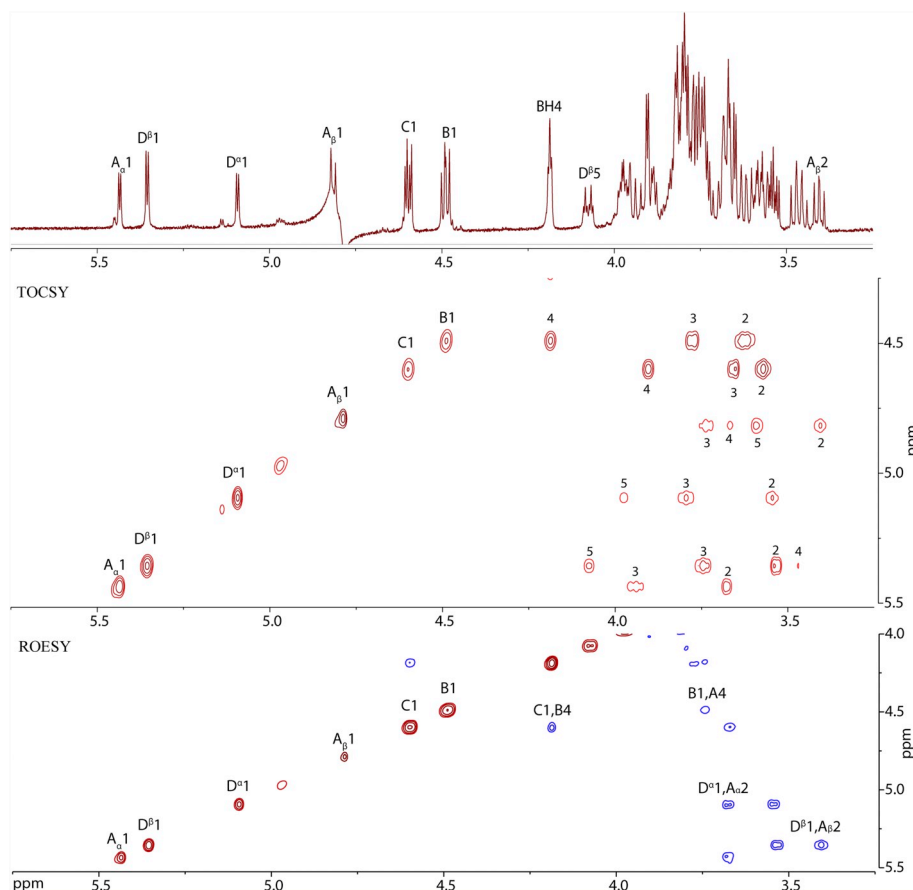
The profiles of the oligosaccharides products were analyzed by HPAEC-PAD on a Dionex ICS-3000 work station (Dionex, Amsterdam, the Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250  $\times$  4 mm; Dionex). The analytical separation was performed at a flow rate of 1.0 mL min $^{-1}$  using a complex gradient of effluents A (100 mM NaOH); B (600 mM NaOAc in 100 mM NaOH); C (Milli-Q water); and D (50 mM NaOAc). The gradient started with 10% A, 85% C, and 5% D in 25 min–40% A, 10% C, and 50% D, followed by a 35-min gradient to 75% A, 25% B, directly followed by 5 min washing with 100% B and reconditioning for 7 min with 10% A, 85% B, and 5% D.

#### 4.5. MALDI-TOF mass spectrometry

Molecular masses of the compounds in the reaction mixtures were determined by MALDI-TOF mass spectrometry on an Axima™ Performance mass spectrometer (Shimadzu Kratos Inc., Manchester, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Ion-gate cut-off was set to  $m/z$  200 and sampling resolution was software-optimized for  $m/z$  1500. Samples were prepared by mixing 1  $\mu\text{L}$  with 1  $\mu\text{L}$  aqueous 10% 2,5-dihydroxybenzoic as matrix solution.

#### 4.6. NMR spectroscopy

The structures of oligosaccharides of interest were elucidated by 1D and 2D  $^1\text{H}$  NMR, and 2D  $^{13}\text{C}$  NMR. A Varian Inova 500 Spectrometer and 600 Spectrometer (NMR center, University of Groningen) were used at probe temperatures of 25 °C with acetone as internal standard (chemical shift of  $\delta$  2.225). The aliquot samples were exchanged twice with 600  $\mu\text{L}$  of 99.9% $_{\text{atom}}$   $\text{D}_2\text{O}$  (Cambridge Isotope Laboratories, Inc., Andover, MA) by freeze-drying, and then dissolved in 0.65 mL  $\text{D}_2\text{O}$ , containing internal acetone. In the 1D  $^1\text{H}$  NMR experiments, the data were recorded at 8 k complex data points, and the HOD signal was suppressed using a WET1D pulse. In the 2D  $^1\text{H}$ - $^1\text{H}$  NMR COSY experiments, data were recorded at 4000 Hz for both directions at 4 k complex data points in 256 increments. 2D  $^1\text{H}$ - $^1\text{H}$  NMR TOCSY data were recorded with 4000 Hz at 30, 60, 100 spinlock times in 200 increments. In the 2D  $^1\text{H}$ - $^1\text{H}$  NMR ROESY, spectra were recorded with 4800 Hz at a mixing time of 300 ms in 256 increments of 4000 complex data points. MestReNova 9.1.0 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process NMR spectra, using Whittaker Smoother baseline correction.



**Fig. 4.** 500-MHz 1D  $^1\text{H}$  NMR spectra of GL3 synthesized by Gtf180- $\Delta\text{N}$  from  $\beta 4'$ -GL, recorded at 25  $^\circ\text{C}$  in  $\text{D}_2\text{O}$ . Anomeric signals of each fraction were labeled according to the legends of the corresponding structures indicated in Scheme 1. The  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of GL3 are listed in Table 2.

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